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The rationale for ovarian cancer prevention is highlighted by the observations that pregnancy and birth control pill use are strongly protective. To achieve a better understanding of the etiology of ovarian cancer, which can then be translated into effective prevention strategies, we have initiated a case-control study that considers genetic susceptibility, epidemiologic risk factors and acquired genetic alterations. Subjects are interviewed in their homes and about 650 cases and 650 controls have been accrued thus far. Blood and cancer samples have been collected and molecular analyses of genetic polymorphisms (BRCA1/2, progesterone receptor) have been performed. An initial ovarian cancer chemoprevention trial with levoneorestrel in chickens demonstrated a protective effect and we have shown that progestin mediated apoptosis in the ovarian epithelium is mediated by transforming growth factor-beta. *In vitro* data has suggested that vitamin D analogues may also represent appealing chemopreventives. We are presently working towards a chemoprevention trial that will incorporate both progestins and vitamin D analogues. We also are exploring novel therapeutic approaches for targeting the progesterone receptor. These studies have the potential to increase our ability to identify high-risk women and to lead to the development of chemoprevention strategies that might decrease mortality from this disease.

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Introduction

Ovarian cancer is the fourth leading cause of cancer deaths among women in the United States. There are three potential approaches to decreasing ovarian cancer mortality: screening and early detection, more effective treatment and prevention. All of these avenues should be explored, but we believe that prevention represents the most feasible approach. The rationale for prevention is derived from epidemiologic studies that have examined the relationship between reproductive history, hormone use and ovarian cancer. It has been convincingly demonstrated that reproductive events which reduce lifetime ovulatory cycles are protective. Although most women are unaware of this protective effect, those who use oral contraceptive pills for more than 5 years or have 3 children decrease their risk of ovarian cancer by greater than 50%. The biological mechanisms that underlie the association between ovulation and ovarian cancer are poorly understood, however.

Our multidisciplinary ovarian cancer research group has been actively involved in studies that seek to elucidate the etiology of ovarian cancer and to translate this knowledge into effective preventive strategies. Joint consideration of genetic susceptibility, reproductive/hormonal and other exposures, acquired alterations in oncogenes and tumor suppressor genes and protective mechanisms such as apoptosis is required to accomplish this goal. We have initiated a molecular epidemiologic study of ovarian cancer in North Carolina that focuses on the identification of genetic polymorphisms that affect susceptibility to ovarian cancer. Over 1,300 subjects have been accrued thus far in this case-control study. We have examined several polymorphisms and also have forged a collaboration with a group in Australia that is also conducting a DOD funded case-control study of ovarian cancer. This will facilitate progress by allowing us to confirm positive results. In addition, we will pool polymorphism data to increase statistical power to examine relationships with less common histologic types (eg. borderline and non-serous) and gene-gene and gene-environment interactions.

We also are actively involved in development of chemopreventive strategies. We have performed a study in primates that suggests that the oral contraceptive has a potent apoptotic effect on the ovarian epithelium, mediated by the progestin component. In addition, in subsequent studies performed *in vitro*, we have induced apoptosis in epithelial cells treated with the progestin levonorgestrel. Progestin mediated apoptotic effects may be a major mechanism underlying the protection against ovarian cancer afforded by OCP use. This forms the basis for an investigation of the progestin class of drugs as chemopreventive agents for epithelial ovarian cancer. Initial studies to test the progestin levonorgestrel in an avian model of ovarian cancer have been undertaken and demonstrated a striking protective effect. In the present study, we are exploring the potential use of vitamin D compounds to enhance the apoptotic effect of progestins on the ovarian epithelium and to enhance the protection against ovarian cancer in the avian model. In addition, we are exploring the molecular pathways (most notably the TGF-beta pathway) that mediate progestin/vitamin D induced apoptosis in the ovarian epithelium. Finally, in an "idea project" we are exploring new pharmacologic approaches to targeting the progesterone receptor for ovarian chemoprevention.

Over the past five years with support from the DOD Ovarian Cancer Research Program we have made considerable progress. This report focuses on the most recent progress from the last year in which we were refunded with a second DOD Ovarian Cancer Program Project.

Body

Epidemiology and Tissue Core and Project 1: Genetic susceptibility to ovarian cancer

With the support of the Department of Defense Ovarian Cancer Research Program we have initiated a molecular epidemiologic study of ovarian cancer to work towards the goal of a better understanding of the etiology of ovarian cancer. Drs. Andrew Berchuck (Gynecologic Oncologist) and Joellen Schildkraut (Epidemiologist) are working together to lead this study. Our initial plan was to accrue frozen tumor tissue and blood from 500 epithelial ovarian cancer cases treated at Duke University, the University of North Carolina at Chapel Hill and East Carolina University. In addition, 500 age and race-matched control subjects were to be accrued and both cases and controls were to be interviewed by telephone regarding known risk factors for ovarian cancer. After funding to support this project was received from the Department of Defense in 1998 with Dr Berchuck as PI, additional funding was received to support this project in the form of an RO1 grant from the NCI with Dr Schildkraut as PI. The additional funding has allowed us to increase the scope of the study such that nurse interviewers are visiting the homes of all the cases and controls to administer the study questionnaire. Research subjects are now accrued from hospitals in a 48 county region of central and eastern North Carolina using a rapid case ascertainment mechanism established through the state tumor registry. Prior to initiating the study, we had to go through the process of IRB approval in each of the various hospitals involved. The second DOD Ovarian Cancer Program Project which began in 2002 provides funding to increase our accrual to 820 ovarian cancer cases and an equal number of controls. Thus far about 650 women with ovarian cancer and 650 age and race-matched controls have been entered in the study and interviewed. The investigators have project meetings every month with all the research staff to review progress and address ongoing issues and at this point we are pleased with the accrual rate and other procedural aspects of the study. We continue to obtain blood specimens on over 99% of our study subjects. All clinical, epidemiologic and molecular data are stored as they are obtained in a computerized database. Of 634 paraffin block requests, 548 have been received thus far. These tissues are being used to assess alterations in cancer causing genes such as p53 and HER-2/*neu*. We are continuing to test the hypothesis proposed in the first DOD program project grant that alterations in specific genes may represent molecular signatures that characterize distinct molecular epidemiological pathways of causation of ovarian cancer.

During the study interview a thorough history of the menstrual cycle and reproductive experiences of the study participants is obtained from each subject assisted by the use a life-time calendar method. In addition, information on oral contraceptives and hormone replacement therapy is obtained. Data on the family history of cancer, other risk factors, and potential confounders is also collected. The interview takes 60-90 minutes to complete. The interactions between the nurses and subjects has been uniformly positive. The women with ovarian cancer are highly motivated to talk about their history and have a high level of interest in supporting a study aimed at increasing our understanding of the causes of ovarian cancer. They greatly appreciate the opportunity to talk with a nurse who is truly interested in hearing all the details of their life experience.

Although most of the genes responsible for dominant hereditary ovarian cancer syndromes (BRCA1/2, MSH2/MLH1) likely have been discovered, there is evidence to suggest that polymorphisms in other genes may also affect cancer susceptibility in a more weakly penetrant fashion. In project 1, we are examining the role of genetic susceptibility in the development of ovarian cancer. These studies focus on genes involved in pathways implicated in the development of ovarian cancer. Since the effect of cancer susceptibility genes may be modified by other genes and exposures, he also will determine whether gene-gene and gene-environment interactions affect ovarian cancer susceptibility. Because of the low

incidence of ovarian cancer, the ability to identify "high risk" subsets of women is critical if we hope to translate our emerging understanding of the etiology of ovarian cancer into effective prevention strategies.

BRCA1/2: Since inherited BRCA1or BRCA2 mutations strikingly increase ovarian cancer risk, polymorphisms in these genes could represent low penetrance susceptibility alleles. Prior studies of the BRCA2 N372H polymorphism suggested that HH homozygotes have a modestly increased risk of both breast and ovarian cancer. We have examined whether BRCA2 N372H or common amino acid-changing polymorphisms in BRCA1 predispose to ovarian cancer in the North Carolina ovarian cancer study. Cases included 312 women with ovarian cancer (76% invasive, 24% borderline) and 401 age- and race-matched controls. Blood DNA from subjects was genotyped for BRCA2 N372H and BRCA1 Q356R and P871L. There was no association between BRCA2 N372H and risk of borderline or invasive epithelial ovarian cancer. The overall odds ratio for HH homozygotes was 0.8 (95% CI = 0.4-1.5) and was similar in all subsets including invasive serous cases. In addition, neither the BRCA1 Q356R (OR = 0.9, 95% CI 0.5-1.4) nor P871L (OR = 0.9, 95% CI 0.6-1.9) polymorphisms were associated with ovarian cancer risk. There was a significant racial difference in allele frequencies of the P871L polymorphism (P = 0.64 in Caucasians, L = 0.76 in African Americans, $p<0.0001$). In this population-based, case-control study, common amino acid changing BRCA1 and 2 polymorphisms were not found to affect the risk of developing ovarian cancer. These results were published this year in Clinical Cancer Research (see references).

MMP1: It has been suggested that the 2G allele of a guanine insertion/deletion promoter polymorphism in the promoter of the matrix metalloproteinase-1 (*MMP1*) gene may increase susceptibility to ovarian cancer. The 2G allele also has been associated with increased *MMP1* expression. We investigated the relationship between the *MMP1* polymorphism and ovarian cancer risk in a large population-based, case-control study. The *MMP1* promoter polymorphism was examined in white blood cell DNA from 311 cases and 387 age-and race-matched controls using a radiolabeled PCR assay. In addition, genotyping of the *MMP1* polymorphism performed in 42 advanced stage invasive serous ovarian cancers was compared to their mean relative *MMP1* expression from Affymetrix microarrays. The 2G allele frequency did not differ significantly between cases (0.49) and controls (0.48) and the distribution of genotypes was in Hardy-Weinberg equilibrium. Using 1G homozygotes as the reference group, neither 2G homozygotes (OR 1.1, 95% CI 0.7-1.7) nor heterozygotes plus 2G homozygotes (OR 0.9, 95% CI 0.7-1.3) had an increased risk of ovarian cancer. There was also no relationship between *MMP1* genotype and histologic grade, histologic type, stage, or tumor behavior (borderline vs. invasive). The mean *MMP1* expression was twice as high in 2G homozygotes relative to 1G homozygotes, but this difference was not statistically significant. The reported association between the *MMP1* promoter polymorphism and ovarian cancer risk is not supported by our data. There was a suggestion that the 2G allele may be associated with higher *MMP1* expression and this finding is worthy of further investigation. These results were published this year in the Journal of the Society for Gynecologic Investigation (see references).

Progesterone receptor: In view of the protective effect of progestins against ovarian cancer, progesterone receptor variants with altered biological activity could affect ovarian cancer susceptibility. A German group reported that an insertion polymorphism in intron G of the progesterone receptor increased ovarian cancer risk by 2.1 fold. It subsequently was shown that this intronic *Alu* insertion is in linkage disequilibrium with polymorphisms in exons 4 and 5. However, several subsequent studies by our group (see references) and others have failed to confirm an association between these polymorphisms and ovarian cancer risk.

More recently sequencing of the progesterone receptor gene has revealed several additional polymorphisms including one in the promoter region (+331G/A). The +331A allele creates a unique transcriptional start site that favors production of the progesterone receptor B (PR-B) isoform. The PR-A

and PR-B isoforms are ligand-dependent members of the nuclear receptor family that are structurally identical except for an additional 164 amino acids at the N-terminus of PR-B, but their actions are distinct. The full length PR-B functions as a transcriptional activator and in the tissues where it is expressed it is a mediator of various responses, including the proliferative response to estrogen or the combination of estrogen and progesterone. PR-A is a transcriptionally inactive dominant-negative repressor of steroid hormone transcription activity that is thought to oppose estrogen-induced proliferation. An association has been reported between the +331A allele and increased susceptibility to endometrial and breast cancers. It was postulated that upregulation of PR-B in carriers of the +331A allele might enhance formation of these cancers due to an increased proliferative response.

In view of the known protective effect of progestins against ovarian cancer, we investigated whether the +331G/A polymorphism in the progesterone receptor promoter affects susceptibility to various histologic types of ovarian cancer in the North Carolina ovarian cancer study (table 1). To decrease the likelihood of false-positive associations, this polymorphism was also examined by collaborators in Australia (Dr. Chenevix-Trench) who are also conducting ovarian cancer molecular epidemiological studies funded by the DOD. Data from the two studies was then pooled to increase statistical power.

The +331G/A single nucleotide polymorphism in the promoter of the progesterone receptor initially was genotyped in samples from the North Carolina Ovarian Cancer Study using a TaqMan assay. Because AA homozygotes were rare, 91 samples in which there was some ambiguity regarding the genotype (AA vs AG) were sequenced for confirmation and in all cases the original genotypes were confirmed. The +331A allele was found in 59/504 (11.7%) Caucasian controls and the distribution of genotypes was in Hardy-Weinberg Equilibrium (χ^2 = 0.391, p = 0.53). Only 1/81 (1.2%) African American controls and none of 67 African American women with ovarian cancer carried the +331A allele. In view of the rarity of the +331A allele in African Americans, these subjects were excluded from analyses of the association with ovarian cancer risk. The +331AA homozygotes were combined with GA heterozygotes in calculating crude and age-adjusted odds ratios (Table 2). The +331A allele was associated with a modest reduction in risk of both borderline tumors and invasive ovarian cancers. Analysis by histologic type revealed that there was a slight trend towards protection against the common serous histologic type (OR = 0.80, 95% CI 0.49–1.29) but there was a more striking protection against endometrioid and clear cell cancers (OR = 0.30, 95% CI 0.09–0.97). These associations were not modified by age, parity, history of oral contraceptive use, body mass index or family history of breast/ovarian cancer.

Samples from the Australian study were genotyped independently and 10.7% of controls were found to carry the +331A allele. The distribution of genotypes in controls was found to be in Hardy Weinberg Equilibrium (χ^2 = 1.231, p = 0.27). A similar protective effect against invasive ovarian cancer was seen in the Australian study, but not against borderline tumors (Table 2). Likewise, the protective effect in the Australian study was most pronounced in the endometrioid and clear cell group (OR = 0.60, 95% CI = 0.25–1.44). The relationship between the +331A allele and the risk of various histologic types of ovarian cancer was analyzed in the combined US and Australian data (Table 3). Again, the most notable finding was a significant association between the +331A allele and decreased risk of both endometrioid and clear cell cases. In combining the two subgroups there was a statistically significant risk reduction (OR = 0.46, 95% CI = 0.23–0.92) (P = 0.027). These cases represent 18% of all ovarian cancers and 21% of the invasive cases.

The association between the +331A allele and endometriosis/infertility was examined because these conditions are known to increase the risk of endometrioid and clear cell ovarian cancers. The rates of self-reported endometriosis and infertility respectively in cases (12.6%, 13.2%) and controls (7.5%, 10.5%) in this study were similar to other reports in the literature. Endometriosis was associated with an increased risk of ovarian cancer (OR 1.76, 95% CI = 1.14-2.72). This was mostly attributable to an

increased risk of endometrioid/clear cell cases (OR = 3.872, 95% CI = 2.09-7.17; non-endometrioid/clear cell cases OR = 1.36, 95% CI = 0.84-2.20). Those who reported a history of infertility due to any cause had a slightly increased risk of ovarian cancer (OR = 1.29, 95% CI = 0.87-1.93; endometrioid/clear cell cases OR = 1.22, 95% CI = 0.59-2.52 and non-endometrioid/clear cell cases OR = 1.31, 95% CI = 0.86-1.99). The +331A allele was also associated with a reduced risk of infertility (OR = 0.37, 95% CI = 0.15–0.94) and to a lesser extent endometriosis (OR = 0.56, 95% CI = 0.24–1.33), but this study is under powered to prove this conclusively.

Although the literature is fraught with false-positive association studies of genetic susceptibility polymorphisms, several features mitigate the likelihood of this in the present study. First, the known protective benefit of progestins against ovarian cancer provides a preexisting biologic plausibility for the observed association. In addition, unlike many polymorphisms that lack known functional significance, the +331A allele increases transcription of PR-B when transfected in an *in vitro* model. Finally, confirmation of the results obtained in North Carolina by the Australian study also is supportive. This data is about to be submitted for publication this month. We plan to continue a close collaborative relationship with the Australian group in the future. The discovery of genetic polymorphisms that affect ovarian cancer risk has the potential to facilitate identification of high-risk women who would be candidates for chemopreventive approaches.

Table 1 - Demographics and pathologic characteristics of cases and controls

	Australian		North Carolina	
	Cases (N=535)	Controls (N=298)	Cases (N=438)	Controls (N=504)
	n	n %	n	n
Age				
Median, (range)	59 (30-95)	50 (30-90)	55 (20-74)	53 (20-75)
Menopause Status				
Pre/peri			166 (38%)	204 (40%)
Post			272 (62%)	300 (60%)
Parity*				
0	71 (20%)		93 (21%)	68 (13%)
1	51 (15%)		73 (17%)	72 (14%)
2	103 (30%)		146 (33%)	210 (42%)
≥3	123 (35%)		126 (29%)	154 (31%)
OC Use*				
Yes	169 (49%)		294 (67%)	349 (69%)
No	179 (51%)		144 (33%)	155 (31%)
Tumor Behavior				
Borderline	87 (16%)		102 (23%)	
Invasive	448 (84%)		336 (77%)	
Tumor Stage**				
1	166 (31%)		160 (37%)	
2	42 (8%)		33 (8%)	
3	276 (52%)		224 (52%)	
4	43 (8%)		14 (3%)	
Tumor Histology				
Serous	318 (59%)		270 (62%)	
Endometrioid	63 (12%)		56 (13%)	
Mucinous	61 (11%)		49 (11%)	
Mixed Cell	36 (7%)		1 (<1%)	
Clear Cell	32 (6%)		23 (5%)	
Other	25 (5%)		39 (9%)	

*Parity and OC use not known for 187 Australian cases and 298 Australian controls

**Stage not known for 8 Australian and 7 NC cases

14 Australian cases under age 30 were excluded from entire analysis b/c no controls were under 30

Table 2 – Association between progesterone receptor polymorphism and risk of invasive and borderline epithelial ovarian tumors

North Carolina Study

	All Cases (N=438)		Controls (N=504)				Invasive Cases (N=336)		Controls (N=504)				Borderline Cases (N=102)		Controls (N=504)			
Genotype	n	(%)	n	(%)	OR*	95%CI	n	(%)	n	(%)	OR*	95%CI	n	(%)	n	(%)	OR*	95%CI
GG	400	(91.3)	445	(88.3)	1.00	Reference	307	(91.4)	445	(88.3)	1.00	Reference	93	(91.2)	445	(88.3)	1.00	Reference
AG	37	(8.4)	58	(11.5)			28	(8.3)	58	(11.5)			9	(8.8)	58	(11.5)		
AA	1	(0.2)	1	(0.2)			1	(0.3)	1	(0.2)			0	(0.0)	1	(0.2)		
AG/AA	38	(8.7)	59	(11.7)	0.72	(0.47 - 1.10)	29	(8.6)	59	(11.7)	0.72	(0.45 - 1.15)	9	(8.8)	59	(11.7)	0.70	(0.33 - 1.49

Australian Study

	All Cases (N=535)		Controls (N=298)				Invasive Cases (N=448)		Controls (N=298)				Borderline Cases (N=87)		Controls (N=298)			
Genotype	n	(%)	n	(%)	OR*	95%CI	n	(%)	n	(%)	OR*	95%CI	n	(%)	n	(%)	OR*	95%CI
GG	483	(90.3)	266	(89.3)	1.00	Reference	407	(90.8)	266	(89.3)	1.00	Reference	76	(87.4)	266	(89.3)	1.00	Reference
AG	48	(9.0)	30	(10.1)			37	(8.3)	30	(10.1)			11	(12.6)	30	(10.1)		
AA	4	(0.7)	2	(0.7)			4	(0.9)	2	(0.7)			0	(0.0)	2	(0.7)		
AG/AA	52	(9.7)	32	(10.7)	0.83	(0.51 - 1.35)	41	(9.2)	32	(10.7)	0.76	(0.46 - 1.27)	11	(12.6)	32	(10.7)	1.18	(0.57 - 2.46

Combined data

	All Cases (N=973)		Controls (N=802)				Invasive Cases (N=784)		Controls (N=802)				Borderline Cases (N=189)		Controls (N=802)			
Genotype	n	(%)	n	(%)	OR*	95%CI	n	(%)	n	(%)	OR*	95%CI	n	(%)	n	(%)	OR*	95%CI
GG	883	(90.8)	711	(88.7)	1.00	Reference	714	(91.1)	711	(88.7)	1.00	Reference	169	(89.4)	711	(88.7)	1.00	Reference
AG	85	(8.7)	88	(11.0)			65	(8.3)	88	(11.0)			20	(10.6)	88	(11.0)		
AA	5	(0.5)	3	(0.4)			5	(0.6)	3	(0.4)			0	(0.0)	3	(0.4)		
AG/AA	90	(9.2)	91	(11.3)	0.78	(0.57 - 1.07)	70	(8.9)	91	(11.3)	0.75	(0.53 - 1.06)	20	(10.6)	91	(11.3)	0.94	(0.56 - 1.57

*ORs are adjusted for age. For combined data ORs are adjusted for study as well.

Table 3 – Association between progesterone receptor polymorphism and risk of invasive and borderline epithelial ovarian tumors by histologic type

	All Cases							Invasive Cases							Borderli				
	GG	AG	A A	AG/AA		OR	(95% CI)	GG	AG	AA	AG/AA		OR	(95% CI)	GG	AG	A A	AG/AA	
Controls	711	88	3	91	(11.3%)	1.00	Reference	711	88	3	91	(11.3%)	1.00	Reference	711	88	3	91	(11.3%
Serous	529	57	2	59	(10.0%)	0.86	(0.61 - 1.23)	427	45	2	47	(9.9%)	0.87	(0.59 - 1.28)	102	12	0	12	(10.5%
Mucinous	99	11	0	11	(10.0%)	0.88	(0.45 - 1.71)	44	3	0	3	(6.4%)	0.54	(0.16 - 1.78)	55	8	0	8	(12.7%
Endometrioid	112	6	1	7	(5.9%)	0.48	(0.22 - 1.08)	106	6	1	7	(6.2%)	0.51	(0.23 - 1.14)	6	0	0	0	(0.0%)
Clear cell	52	3	0	3	(5.5%)	0.44	(0.14 - 1.46)	50	3	0	3	(5.7%)	0.46	(0.14 - 1.52)	2	0	0	0	(0.0%)
Endo + clear	164	9	1	10	(5.7%)	0.46	(0.23 - 0.92)	156	9	1	10	(6.0%)	0.49	(0.25 - 0.97)	8	0	0	0	(0.0%)
Mixed	33	3	1	4	(10.8%)	1.07	(0.34 - 3.35)	30	3	1	4	(11.8%)	1.07	(0.34 - 3.35)	3	0	0	0	(0.0%)
Other	58	5	1	6	(9.4%)	0.79	(0.33 - 1.91)	57	5	1	6	(9.5%)	0.81	(0.34 - 1.95)	1	0	0	0	(0.0%)

*Ors are of being a case for the AG/GG compared to the reference group GG and are adjusted for age and study

**Sample size too small to calculate

Project 2: Chemoprevention of ovarian cancer

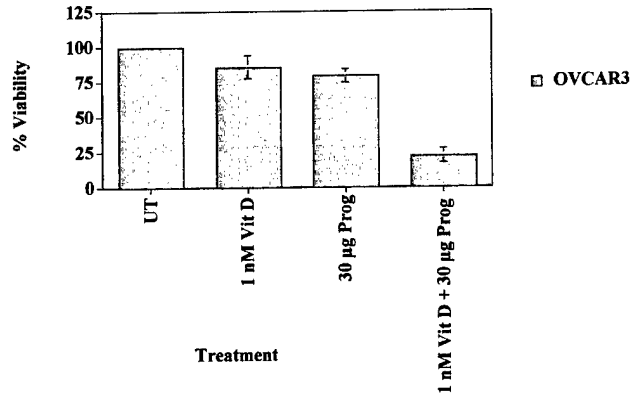
Project 2 is under the direction of Gustavo Rodriguez, M.D. (Gynecologic Oncologist). The prevention strategy outlined in our proposal focuses on the potential use of a combined approach incorporating both progestins and vitamin D analogues. The progestagenic hormonal milieu of pregnancy and oral contraceptives is associated with protection against ovarian cancer; and progestins have a potent apoptotic effect on ovarian epithelial cells. In addition, the increasing incidence of ovarian cancer in northern latitudes suggests a role for sunlight/vitamin D deficiency in the etiology of ovarian cancer. In this regard, vitamin D decreases proliferation of ovarian cancer cells has been shown to induce apoptosis and have chemopreventive properties in various cell types.

With regard to cancer prevention, the apoptosis pathway is one of the most important *in vivo* mechanisms that functions to eliminate cells that have sustained DNA damage and which are thus prone to malignant transformation. In addition, a number of well-known chemopreventive agents have been demonstrated to activate the apoptosis pathway in the target tissues that they protect from neoplastic transformation. We have performed a study in primates that suggests that the oral contraceptives (OCs) have a potent apoptotic effect on the ovarian epithelium, mediated by the progestin component. In addition, in subsequent studies performed in vitro, we have induced apoptosis in transformed, immortalized, cultured human ovarian epithelial cells treated with the progestin levonorgestrel. This suggests that progestins may have a direct apoptotic effect on the ovarian epithelium. The finding that progestins activate this critical pathway in the ovarian epithelium, the site where ovarian cancers arise, makes it likely that progestin mediated apoptotic effects are a major mechanism underlying the protection against ovarian cancer afforded by routine OC use. This forms the basis for an investigation of the progestin class of drugs as chemopreventive agents for epithelial ovarian cancer.

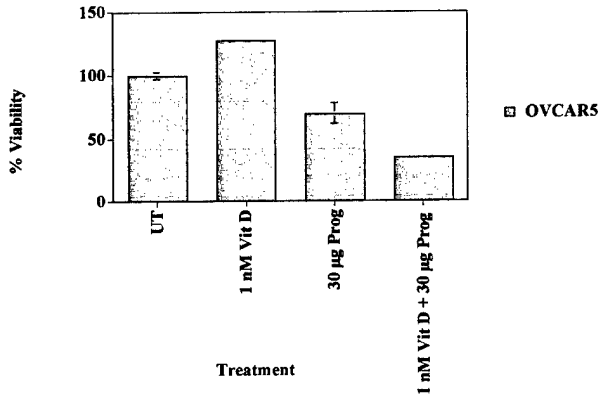
The studies outlined in our prevention grant are designed to add further support to notion that progestins and vitamin D analogues are potent apoptotic agents on human ovarian epithelial cells, and to directly test the hypothesis in an animal model these agents confer preventive effects against ovarian cancer. These aims in the grant are: (1) to evaluate the apoptotic effect of progestins and vitamin D analogues on the human ovarian epithelium *in vivo*, (2) elucidate the molecular mechanisms by which they induce apoptosis in ovarian epithelial cells, and (3) to directly test the hypothesis that progestins/vitamin D analogues confer preventive effects against ovarian cancer in a chemoprevention trial in the chicken, the only animal species with a high incidence of ovarian cancer.

After this DOD ovarian cancer program project was submitted, Dr Rodriguez moved to Northwetern University (Evanston campus) in Chicago. Transfer of the funds from Duke to Northwestern took more than 6 months, but despite a late start excellent progress has been achieved on this project. The *in vitro* conditions have been defined in which progesterone and vitamin D consistently and markedly inhibit growth of ovarian cancer cell lines (see figures below). Moreover, our data suggest that the combination of Vitamin D and progesterone have a more potent effect than either agent administered alone. We are in the process of doing assays to determine whether the effect is additive or synergistic. The figures below demonstrates a marked impact on cell viability when the two agents are combined, and administered at a dosage that has a marginal impact for each agent given alone.

Effect of Vitamin D and Progesterone
on OVCAR3 Cells



Effect of Vitamin D and Progesterone
on OVCAR5 Cells



Now that we can reliably impact cell growth, apoptosis/viability, we have studies underway to assess the impact of these hormones on the apoptotic and TGF-beta signaling pathways. We will also then examine the relative importance of the Progesterone receptor (PR) A and PRB isoforms for mediating the effect of vitamin D and progestins on cells derived from the ovarian epithelium. Studies are underway/planned in not only ovarian cancer cell lines, but also normal ovarian surface epithelial cells (NOSE) and transformed NOSE. We are also doing the preliminary work for a collaboration with Elise Kohn at NCI to examine (using phosphoproteomic arrays) functional proteins from the TGF-beta and apoptotic pathways in ovarian epithelium. We will also be doing laser capture on the ovarian epithelium for expression

microarray studies to gain insight/understand the complex signaling events induced by progestins and vitamin D analogues.

With regard to the chicken chemoprevention study, we have identified a flock and plan to commence this trial later in 2004. In addition, we have carefully studied flocks of birds treated under different conditions, leading to important insights regarding the incidence of cancer relative to bird strain, age, and flock handling. We can now use this data to better design the upcoming study. In addition, through a collaboration with Bill Cliby at the Mayo CLinic, we have preliminary data that the genomic fingerprint differs between oviductal and ovarian primary lesions in the chicken. If confirmed, this will facilitate analysis of the outcome (tumor incidence) data in the chicken trial.

Idea Project: Probing the mechanism(s) of crosstalk between estrogen and progesterone signaling pathways: A first step in the search for novel chemopreventatives

Donald P. McDonnell, Ph.D.

One of the primary functions of progestins is to oppose the mitogenic action of estradiol in the uterus. While the physiological activities of progesterone are clearly defined, the mechanism by which it exerts its anti-estrogenic actions is less clear. Furthermore, it is unclear in ovarian epithelial cells if the chemoprotective effect of progestins are related to its ability to attenuate estrogen signaling or if an independent activity is responsible. Consequently, a primary focus of our research efforts of late has been geared towards an understanding of the basic mechanism by which progesterone and its receptors crosstalk with the estrogen signaling pathways in breast, uterine and ovarian cancer cells. This, we believe, will lead to a better understanding of the positive actions of progestins in these targets.

Previously, it has been demonstrated that the biological actions of progestins are mediated by either of two forms of the progesterone receptor (PRA or PRB). Furthermore, it was demonstrated in reconstituted transcription systems that agonist or antagonist-activated PRA, but not PRB, could efficiently suppress estrogen signaling. Since agonists and antagonists recruit different protein complexes, the mechanisms by which these two classes of ligands mediate transrepression are likely to be distinct. In our latest studies, we show that in T47D breast cancer cells, which constitutively express PRA, PRB and ER, that estrogen-stimulated transcription of several estrogen responsive genes are inhibited upon co-treatment with PR ligands. In BG-1 ovarian cancer cells, we observe that this inhibitory activity is defective and the mitogenic actions of estrogen are not impeded by progestins. We will extend these studies to other ovarian cell lines in the near future to see if this is a common feature of transformed ovarian epithelial cells. The goal is to identify why most ER/PR containing cells recognize progestins as "antiestrogens" whereas this is apparently not the case in ovarian cells. In the meantime, we continue to explore the cross-talk between ER/PR signaling in cells where they show demonstrable coupling. These studies have led to the identification of two distinct response patterns represented by estrogen responsive genes which are transrepressed by a) both PR agonists and antagonists and b) agonists but not antagonists. Mechanistic studies using transfected cell systems have revealed that PRA can not only transrepress the activation functions of ER, but can also inhibit the autonomous transcriptional activity of p160 co-activators that mediate ER transcriptional activity. The molecular basis for these differences and their significance in ovarian cancer cells is a research priority in the group.

Key research accomplishments

1) We have accrued over 1,300 subjects to a prospective, population-based, case-control study of ovarian cancer in North Carolina. Blood and tissue samples and epidemiologic data have been accrued as well. Analyses of genetic susceptibility polymorphisms and molecular epidemiologic signatures are ongoing. It appears that the +331G/A polymorphism in the progesterone receptor is protective against endometrioid/clear cell cancer.

2) We have shown that progestins markedly activate TGF-β signaling pathways in the ovarian epithelium in primates, and that these effects are highly associated with apoptosis. We are now performing studies *in vitro* designed to characterize the complex biologic effects of progestins and vitamin D analogues on apoptotic and TGF-β signaling pathways in ovarian epithelial cells. These findings will provide guidance in conducting a chemopreventive trial in chickens with these agents.

Reportable outcomes

1) The +331G/A polymorphism may be protective against endometrioid and clear cell ovarian cancers.

2) Combinations of progestins and vitamin D may act in an additive fashion to decrease growth of ovarian cancer cells.

Conclusions

The studies initiated by our program will enable us to define more homogeneous subsets of ovarian cancer based on epidemiologic and molecular characteristics, to identify women who are at increased risk for this disease and to develop chemopreventive strategies designed to decrease ovarian cancer incidence and mortality. We anticipate that much of our data will grow to maturity in the coming few years with continued support from the DOD Ovarian Cancer Research Program.

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Appendices

Polymorphisms in BRCA1 and BRCA2 and Risk of Epithelial Ovarian Cancer

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ABSTRACT

Purpose: **Because inherited BRCA1or BRCA2 mutations strikingly increase ovarian cancer risk, polymorphisms in these genes could represent low penetrance susceptibility alleles. Previous studies of the BRCA2 N372H polymorphism suggested that HH homozygotes have a modestly increased risk of both breast and ovarian cancer. We have examined whether BRCA2 N372H or common amino acid-changing polymorphisms in BRCA1 predispose to ovarian cancer.**

Experimental Design: **A population-based, case control study of ovarian cancer was performed in North Carolina. Cases included 312 women with ovarian cancer (76% invasive and 24% borderline) and 401 age- and race-matched controls. Blood DNA from subjects was genotyped for BRCA2 N372H and BRCA1 Q356R and P871L.**

Results: **There was no association between BRCA2 N372H and risk of borderline or invasive epithelial ovarian cancer. The overall odds ratio (OR) for HH homozygotes was 0.8 [95% confidence interval (CI) = 0.4–1.5] and was similar in all subsets, including invasive serous cases. In addition, neither the BRCA1 Q356R (OR = 0.9, 95% CI 0.5–1.4) nor P871L (OR = 0.9, 95% CI 0.6–1.9) polymorphisms were associated with ovarian cancer risk. There was a significant racial difference in allele frequencies of the P871L polymorphism ($P = 0.64$ in Caucasians, L = 0.76 in African-Americans, $P < 0.0001$).**

Conclusions: **In this population-based, case control study, common amino acid changing BRCA1 and 2 polymorphisms were not found to affect the risk of developing ovarian cancer.**

INTRODUCTION

Germ-line mutations in the BRCA1 or BRCA2 genes strikingly increase lifetime risks of ovarian cancer (10–15% in BRCA2 carriers and 15–30% in BRCA1 carriers; Refs. 1–3). Highly penetrant germ-line BRCA mutations are rare, however, and are carried by <0.5% of individuals in most populations, with the notable exception of Ashkenazi Jews (2.5% carrier rate; Ref. 4). The ability to identify BRCA mutation carriers is an important advance, because these women can consider prophylactic oophorectomy and other approaches aimed at decreasing ovarian cancer mortality (5), but because BRCA mutations are rare, the overall impact on mortality inevitably will be small.

BRCA1 and 2 were identified by focusing on families with multiple early onset breast and/or ovarian cancers, and it is estimated that ~10% of ovarian cancers are attributable to high penetrance mutations in these genes (6–8). However, studies that have compared the incidence of ovarian cancer in identical and fraternal twins have estimated that 22% of cases have a heritable component (9). Although other unknown high penetrance genes may exist, there may be weakly penetrant functional genetic polymorphisms that contribute to the burden of ovarian cancers classified as "sporadic" based on the lack of other cases in a pedigree.

Because BRCA1 and BRCA2 mutations strikingly increase ovarian cancer risk, polymorphisms in these genes are logical candidates in seeking to identify low penetrance susceptibility alleles. In the *BRCA2* gene, N372H is the only amino acid-changing polymorphism with a rare allele frequency of >6%, and an increased risk of breast cancer (crude OR[2] = 1.31) has been reported in HH homozygotes in a large case control study (10). Subsequently, an Australian group also reported that homozygosity for the H allele was associated with increased risks of both breast cancer (OR = 1.42; 95% CI 1.00–2.02; Ref. 11) and ovarian cancer (OR = 1.36; 95% CI 1.04–1.77; Ref. 12). There are five amino acid changing polymorphisms in BRCA1 with rare allele frequencies >5% (Q356R, P871L, E1038G, K1183R, and S1613G; Ref. 13). Some, but not all, previous reports have suggested that these polymorphisms might affect ovarian cancer risk (13–16), but none of these data were derived from population-based case control studies of ovarian cancer. These polymorphisms, with the exception of Q356R, are in significant linkage disequilibrium, and the effect of all of these on ovarian cancer risk can be ascertained by considering only Q356R and P871L (13, 14).

In this study, we examine the association of ovarian cancer risk with BRCA2 N372H and BRCA1 Q356R and P871L in a population-based, case control study in North Carolina.

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[2] The abbreviations used are: OR, odds ratio; CI, confidence interval; NCOC, North Carolina Ovarian Cancer; HCFA, Health Care Financing Administration; dNTP, deoxynucleotide triphosphate.

MATERIALS AND METHODS

Subject Identification and Interview. Study subjects are enrolled through the NCOC study, an ongoing population-based, case control study of newly diagnosed epithelial ovarian cancer. Cases are identified through the North Carolina Central Cancer Registry, a statewide population-based tumor registry, using rapid case ascertainment. Eligible cases are women diagnosed with epithelial ovarian cancer since January 1, 1999, aged 20–74 years, who had no previous history of ovarian cancer, and resided in a 48 county area of North Carolina. Physician permission was obtained before any eligible case was contacted by the study staff. All cases are confirmed by standardized pathological review by the study pathologist. Cases diagnosed with primary epithelial ovarian cancer, either invasive, or of low malignant potential are eligible for the study. The response rate among eligible cases was 85%. Reasons why some patients were not interviewed included death (3.3%), debilitating illness (1.7%), patient refusal (5.4%), physician refusal (5.4%), or the inability to locate the woman (4.4%).

Population-based controls were identified from the same 48 county region as the cases and were frequency matched to the ovarian cancer cases on the basis of race (African-American *versus* non-African-American) and age (5-year age categories) using list-assisted random digit dialing. Although HCFA records (women 65–74 only) lists were used early in the data collection period, enrollment of control women using this method was hindered because of the lack of telephone numbers on the HCFA computer tapes, and the use of HCFA lists was suspended. Potential controls were screened for eligibility and required to have at least one intact ovary. Seventy-three percent of controls identified by random digit dialing who passed the eligibility screening agreed to be contacted and sent additional study information. Among those sent additional study information, the response rate was 70%. The response rate was 39% for HCFA controls from the first 76 eligible subjects identified before the use of HCFA tapes was suspended. The response rate among eligible controls was 67%. Reasons for nonparticipation were refusal (26.1%) and the inability to locate the woman (6.7%). For the purposes of this report, we restricted study subjects to those who either were Caucasian or African-American.

In-person Interview. All women gave written informed consent at the time of the interview. The study protocol was approved by the Duke University Medical Center Institutional Review Board and the human subjects committees at each of the hospitals where cases were identified.

Cases and controls were interviewed in person by trained nurse interviewers, usually in the home of the study subject. A 90-min questionnaire was administered to obtain information on known and suspected ovarian cancer risk factors, including family history of cancer in first and second degree relatives, menstrual characteristics, pregnancy and breastfeeding history, hormone use, and lifestyle characteristics, such as smoking history, alcohol consumption, physical activity, and occupational history. Additionally, anthropometric descriptors (height, weight, waist, and hip circumference) are measured, and a blood sample (30 ml) is collected from each participant at the time of the interview.

Blood Processing. Within 48 h, all blood samples are centrifuged, and the buffy coat, RBCs, and plasma are separated. Germ-line DNA was extracted from peripheral blood using PureGene DNA isolation reagents, according to manufacturer's instructions (Gentra Systems, Minneapolis, MN).

BRCA2 N372H Polymorphism. Direct sequencing of the exon 10 region containing the polymorphic (A→ C) base was performed on extracted leukocyte DNA. A 50-μl PCR reaction was performed using forward primer 5′-CTG AAG TGG AAC CAA ATG ATA CTG A-3′ and reverse primer 5′-AGA CGG TAC AAC TTC CTT GGA GAT-3′, 0.5 ng/μl DNA, 0.5 nmol/liter forward primer, 0.5 nmol/liter reverse primer, 0.2 nmol/liter dNTP, 1.5 mmol/liter $MgCl_2$, (Life Technologies, Inc.), 1 × Amplitaq Gold PCR buffer II, and 0.025 units/μl AmpliTaq Gold DNA polymerase (Roche, Branchburg, NJ). PCR conditions consisted of an initial denaturing step at 95°C for 12 min, 32 cycles of 94°C for 60 s, 55°C for 60 s, and 72°C for 1 min, an extension step at 72°C for 10 min, and then held at 4°C until further processing. Samples were purified using QIAquick 96 vacuum filter plates (Qiagen, Germantown, MD) and finally eluted in 150 μl of 10 mM Tris-Cl (pH 8.5). A sequencing reaction was performed using 1 μl of purified product and 4.4 pmoles of unlabeled forward primer in a BigDye Terminator Cycle Sequencing Reaction as described by the supplier (Applied Biosystems, Foster City, CA). Samples were analyzed on the ABI 377 autosequencer, and sequences were analyzed with Genescan software (Perkin-Elmer).

BRCA1 P871L Polymorphism. Allelic discrimination was performed using the MGB primer/probe TaqMan assay on the ABI Prism 7700. Each 20-μl PCR reaction contained 18 pmoles of forward primer 5′-GGT TTC AAA GCG CCA GTC AT-3′, 18 pmoles of reverse primer 5′-CAC ATT CCT CTT CTG CAT TTC CT-3′, 4 pmoles of "proline" probe 5′-VIC-TGC TC*C* GTT TTC AAA-3′, 4 pmoles of the "leucine" probe 5′-6FAM-TTG CTC *T*GT TTT CAA AT-3′, 10 μl of 2 × TaqMan universal master mix without Amp erase UNG (Applied Biosystems), and 25 ng of extracted leukocyte DNA. Cycling conditions were 95°C for 10 min followed by 40 cycles of 92°C × 15 s and 60°C × 60 s. Samples were then read in 96-plate format in the ABI Prism 7700 and analyzed using the ABI Prism 7700 allelic discrimination software.

A fraction of samples were subjected to sequencing to confirm results obtained using the TaqMan assay. A 50-μl PCR reaction was performed using forward primer 5′-CCC AAG GGA CTA ATT CAT GG–3′ and reverse primer 5′-TCT GCA TTT CCT GGA TTT GA–3′, 0.5 ng/μl genomic DNA, 0.5 nmol/liter forward primer, 0.5 nmol/liter reverse primer, 0.2 mmol/liter dNTP, 1.5 mmol/liter $MgCl_2$ (Life Technologies, Inc.), 1 × Life Technologies, Inc. PCR buffer (part #Y02028), and 0.025 unit/μl TaqDNA polymerase (Life Technologies, Inc.; catalogue no. 10342-020). PCR conditions consisted of an initial denaturing step at 95°C for 3 min, 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, an extension step at 72°C for 10 min. Samples were held at 4°C until purified using QIAquick 96 vacuum filter plates (Qiagen) and finally eluted in 150 μl of 10 mM Tris-Cl (pH 8.5). A sequencing reaction was performed using 1 μl of purified product and 4.4 pmoles of unlabeled forward primer in a BigDye Terminator Cycle Sequencing Reaction as described by the supplier (Applied Bio-

systems). Samples were analyzed on the ABI 3100, and sequences were determined with Genescan software (Perkin-Elmer).

BRCA1 Q356R Polymorphism. PCR was performed using the forward primer 5′-GGA CTC CCA GCA CAG AAA AA-3′ and reverse primer 5′-TCC CCA TCA TGT GAG TCA TC-3′. The reaction was conducted in a final volume of 15 μl containing 0.5 ng/μl genomic DNA, 0.5 nmol/liter forward primer, 0.5 nmol/liter reverse primer, 0.2 mmol/liter dNTP, 1.5 mmol/liter $MgCl_2$ (Life Technologies, Inc.), 1 × Life Technologies, Inc. PCR buffer (part #Y02028), and 0.025 units/μl TaqDNA polymerase (Life Technologies, Inc.; cat. #10342-020). PCR conditions consisted of an initial denaturing step at 95°C for 3 min, 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, an extension step at 72°C for 10 min, then at 4°C until digested. A digest of the amplicon was performed by combining 15 μl of the PCR amplification, 2 μl of 10 × NEB Buffer 4, and 10 units of *Alu*NI (New England Biolabs, Beverly, MA) in a final volume of 20 μl. Samples were incubated at 37°C for 4.5 h and analyzed immediately on a 2% agarose gel. The undigested arginine (R) allele can be seen as a band at 211 bp, whereas the glutamine (Q) allele is represented by the digestion products at 134 and 77 bp.

A fraction of samples were subjected to sequencing to confirm results obtained using the restriction fragment length analysis described above. For sequencing, completed 50-μl PCR amplifications were purified using QIAquick 96 vacuum filter plates (Qiagen) and reconstituted in 150 μl of elution buffer. A sequencing reaction was performed using 1 μl of purified product and 4.4 pmoles of unlabeled forward primer in a BigDye Terminator Cycle Sequencing Reaction as described by the supplier (Applied Biosystems). Samples were analyzed on the ABI 3100, and sequences were determined with Genescan software (Perkin-Elmer).

Statistical Analysis. The genotype data were tested for Hardy-Weinberg Equilibrium using the χ^2 goodness of fit test. ORs and 95% CIs for the association between the BRCA1 and BRCA2 polymorphisms and epithelial ovarian cancer were calculated for all cases, all serous cases, invasive cases, and invasive serous cases. Logistic regression analysis was used to compute adjusted ORs accounting for age, race, and other potential confounders. Crude ORs are reported as well as adjusted ORs from a multivariable logistic regression model, which included race and age and other potential confounders. Menopausal status, tubal ligation, oral contraceptive use, family history of breast or ovarian cancer in first and second degree relatives, and parity were individually tested to determine whether they changed the crude OR by 10%. All calculations were performed with SAS 8.0 (SAS Institute, Inc., Cary, NC) using unconditional logistic regression. With our current sample size of ~300 cases and 400 controls, we have 80% power to detect an OR of ≥1.6 with type 1 error level equal to 0.05 for BRCA2 N372H and BRCA1 P871L polymorphisms. Because of the lower prevalence of the BRCA1 Q356 R allele, we have 80% to detect an OR of 1.9.

RESULTS

The distributions of epidemiological risk factors for the cases and controls are shown in Table 1. Cases and controls are similar in age and race, with 11% of cases and 13% of controls being self-reported as African-American. The distributions of menopausal status, oral contraceptive use, and family history of breast or ovarian cancer are also similar. Controls tended to have had more pregnancies than cases and were more likely to have had tubal ligations (33 *versus* 24%). The distributions of ovarian tumor characteristics, including stage, histology, and tumor behavior, are noted in Table 1. Over half of cases were diagnosed with stage III/IV disease, 76% had invasive cancers, and 59% were of the serous histological subtype.

Table 1 Demographic and pathologic characteristics of cancer cases and controls

	Cases (n = 312) n (%)	Controls (n = 401) n (%)
Age, years		
20–54	155 (50)	210 (52)
55–75	157 (50)	191 (48)
Race		
Caucasian	278 (89)	349 (87)
African-American	34 (11)	52 (13)
Menopause status		
Pre/peri	121 (39)	165 (41)
Post	191 (61)	236 (59)
Ever-smoked 100 cigarettes	147 (47)	201 (50)
OC use (years)		
Nonusers	108 (35)	133 (34)
<2	66 (21)	64 (16)
2–5	59 (19)	81 (20)
>5	76 (25)	118 (29)
Unknown	3	5
Pregnancies resulting in live birth		
0	60 (19)	54 (13)
1	58 (19)	66 (16)
2	106 (34)	147 (37)
3	52 (17)	84 (21)
4+	36 (12)	50 (12)
Had tubal ligation	75 (24)	132 (33)
BMI		
15–23	70 (23)	99 (26)
23–26	69 (22)	95 (24)
26–30	65 (21)	94 (24)
>30	105 (34)	98 (25)
Family history of breast or ovarian cancer in a 1° relative		
Yes	52 (17)	66 (16)
No	260 (83)	355 (84)
Tumor behavior		
Borderline	75 (24)	
Invasive	237 (76)	
Tumor stage[a]		
I/II	132 (43)	
III/IV	178 (57)	
Tumor histology		
Serous	185 (59)	
Endometrioid	38 (12)	
Mucinous	40 (13)	
Adenocarcinoma	22 (7)	
Clear cell	15 (5)	
Other	12 (4)	

[a] Two missing tumor stage. Three cases and 15 controls missing body mass index.

BRCA2 N372H Polymorphism. Genotyping was performed using automated DNA sequencing in 312 cases and 398

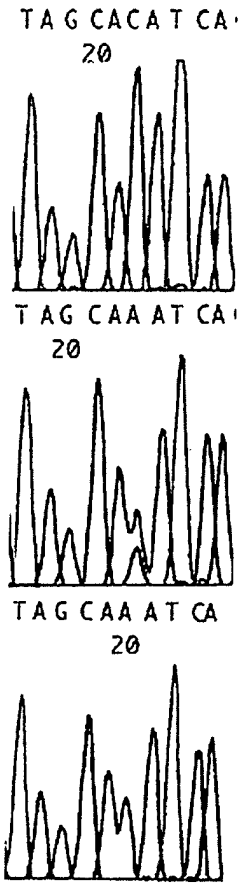

Fig. 1 DNA sequencing of BRCA2 N372H polymorphism. *Top panel,* HH homozygote; *middle panel,* NH heterozygote; *bottom panel,* NN homozygote.

Table 2 ORs and 95% CIs for the association between ovarian cancer risk and BRCA1 and BRCA2 polymorphisms

Genotype	Cases *n* (%)	Controls *n* (%)	Adjusted OR[a]	95% CI
BRCA2 N372H				
NN	169 (54)	227 (57)	1.0	(Referent)
NH	128 (41)	146 (37)	1.2	(0.8–1.6)
HH	15 (5)	25 (6)	0.8	(0.4–1.5)
NH/HH	143 (46)	171 (43)	1.1	(0.8–1.5)
BRCA1 P871L				
PP	127 (42)	141 (36)	1.0	(Referent)
PL	123 (40)	174 (45)	0.8	(0.6–1.1)
LL	55 (18)	73 (19)	0.9	(0.6–1.5)
PL/LL	178 (58)	247 (64)	0.8	(0.6–1.1)
BRCA1 Q356R				
QQ	275 (91)	344 (90)	1.0	(Referent)
QR	27 (9)	39 (10)	0.9	(0.5–1.4)
RR	1 (0)	1 (0)	1.3	(0.1–20.2)
QR/RR	28 (9)	40 (10)	0.9	(0.5–1.4)

[a] Adjusted for race and age.

Table 3 ORs and 95% CIs for the association of serous ovarian cancer risk and BRCA1 and BRCA2 polymorphisms

Genotype	Cases *n* %	Controls *n* %	Adjusted OR[a]	95% CI
BRCA2 N372H				
NN	100 (54)	227 (57)	1.0	(Referent)
NH	76 (41)	146 (37)	1.2	(0.8–1.7)
HH	9 (5)	25 (6)	0.8	(0.4–1.8)
NH/HH	85 (46)	171 (43)	1.1	(0.8–1.6)
BRCA1 P871L				
PP	60 (39)	141 (36)	1.0	(Referent)
PL	64 (42)	174 (45)	0.9	(0.6–1.3)
LL	30 (19)	73 (19)	1.0	(0.6–1.7)
PL/LL	94 (61)	247 (64)	0.9	(0.6–1.3)
BRCA1 Q356R				
QQ	167 (92)	344 (90)	1.0	(Referent)
QR	14 (8)	39 (10)	0.7	(0.4–1.4)
RR	0 (0)	1 (0)		Inestimable
QR/RR	14 (8)	40 (10)	0.7	(0.4–1.4)

[a] Adjusted for race and age.

controls (Fig. 1). Confirmatory sequencing performed in a random 10% subset of cases and controls showed 97.2% (70 of 72) agreement. Among control women, the distribution of genotypes was found to be in Hardy-Weinberg Equilibrium (χ^2 = 0.055, P = 0.8). The frequency of the rare H allele was 0.246 among all controls and 0.257 among Caucasian controls. There was no significant association between the N372H polymorphism and risk of ovarian cancer using either a recessive or codominant model (Table 2). Overall, 5% of invasive and borderline ovarian cancer cases and 6% of controls were homozygous for the rare H allele (OR = 0.8, 95% CI 0.4–1.5). Similar ORs were seen in the subgroups with invasive cancers (OR = 0.8, 95% CI 0.4–1.7), all serous tumors (OR = 0.8, 95% CI 0.4–1.8; Table 3), and invasive serous cancers (OR = 0.7, 95% CI 0.3–1.9).

BRCA1 P871L Polymorphism. Genotyping was performed in 305 cases and 388 controls using a TaqMan assay (Fig. 2). DNA sequencing was performed in 14 samples, including both homozygotes and heterozygotes, and there was complete concordance with the genotypes obtained using the TaqMan assay. Genotype frequencies differed dramatically between Caucasian and African-American control women. Among 337 Caucasian controls, there were 137 PP (41%), 158 PL (47%), and 42 LL (12%), whereas among 51 African-Americans, there were 4 PP (8%), 16 PL (31%), and 31 LL (61%). Frequencies of the P and L alleles were 0.64 and 0.36 among Caucasians and 0.24 and 0.76 among African-Americans ($P < 0.0001$). In both groups of control women, the distribution of genotypes was found to be in Hardy-Weinberg Equilibrium (Caucasians: χ^2 = 0.117, P = 0.7; African-Americans: χ^2 = 0.84, P = 0.36). There was no significant association between the P871L polymorphism and risk of ovarian cancer using a recessive or codominant model in the entire group (Table 2) or in either racial group alone (data not shown). Overall, 18% of invasive and borderline ovarian cancer cases and 19% of controls were homozygous for the L allele (OR = 0.9, 95% CI 0.6–1.5). Similar ORs were seen in the subgroups with invasive cancers (OR = 0.8, 95% CI 0.4–1.3), all serous tumors (OR = 1.0, 95% CI 0.6–1.7; Table 3), and invasive serous cancers (OR = 0.8, 95% CI 0.4–1.5).

BRCA1 Q356R Polymorphism. Genotyping was performed in 303 cases and 384 controls using restriction fragment-

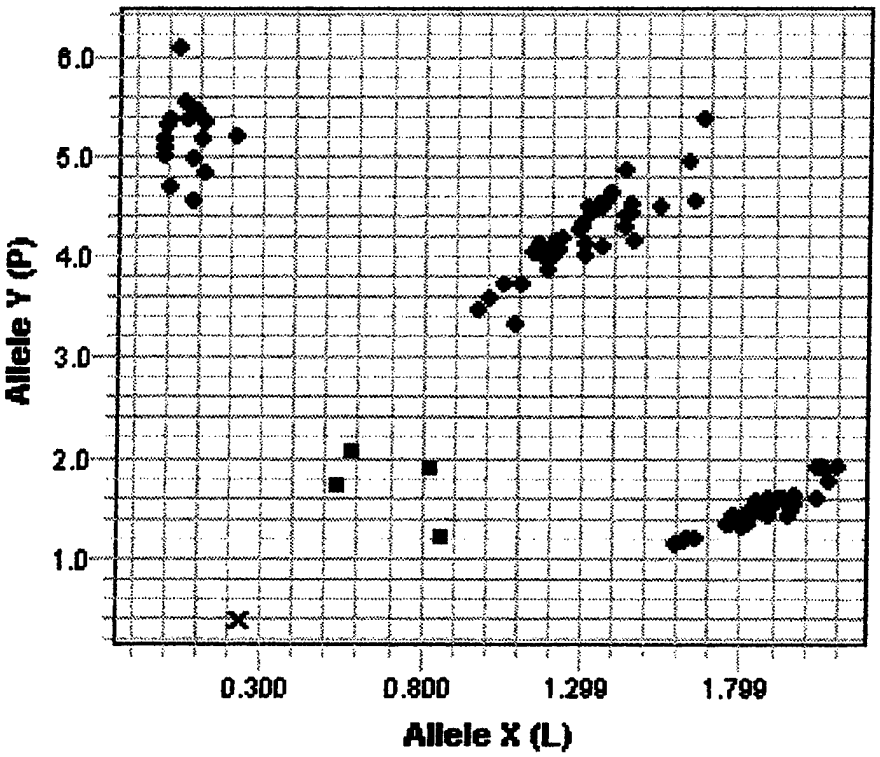

Fig. 2 BRCA1 P871L genotyping using TaqMan assay. PP homozygotes (*blue*), LL homozygotes (*red*), PL heterozygotes (*green*), and water controls (■). The black X represents an uninterpretable outlier.

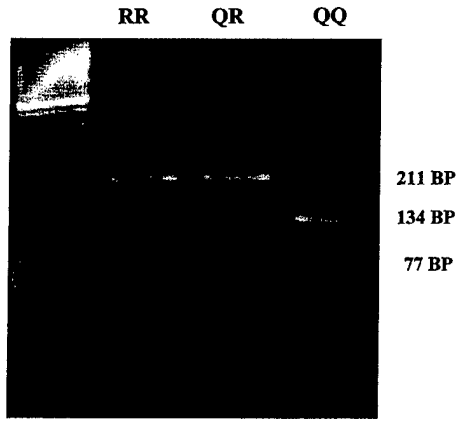

Fig. 3 BRCA1 Q356R genotyping using restriction fragment length analysis with PCR products resolved on a 2% agarose gel. The undigested R allele is represented as a 211-bp band and the Q allele as digest fragments of 134 and 77 bp. *Lane 1,* RR homozygote; *Lane 2,* QR heterozygote; *Lane 3,* QQ homozygote.

length analysis (Fig. 3). The R allele was both rarer and more prone to be incorrectly scored, as a band corresponding to the R allele could represent the misleading remnant of incomplete digestion. In contrast, the Q allele, being a digest product, was unlikely to be present falsely. Confirmatory DNA sequencing was performed in 95 cases, including all 54 samples that were homozygous or heterozygous for the R allele. In only one of 95 samples sequenced was the genotype changed yielding an accuracy of 98.9%. Among control women, the distribution of genotypes was found to be in Hardy-Weinberg equilibrium ($\chi^2 = 0.009$, $P = 0.928$). There was no significant association between the Q356R polymorphism and risk of ovarian cancer (Table 2). The rates of heterozygosity and homozygosity for the R allele were 9% and <1% in ovarian cancer cases and 10% and <1% in controls. Using a codominant model, the combined OR was 0.9 (95% CI 0.5–1.4). Similar ORs were seen in the subgroups with invasive cancers (OR = 0.8, 95% CI 0.4–1.4), all serous tumors (OR = 0.7, 95% CI 0.4–1.4; Table 3), and invasive serous cancers (OR = 0.7, 95% CI 0.3–1.4).

None of the BRCA1 or BRCA2 polymorphisms were associated with ovarian cancer risk when cases were stratified by race, age of onset, parity, history of oral contraceptive use, or family history of breast/ovarian cancer (data not shown).

DISCUSSION

Previous reports have examined the relationship between polymorphisms in genes such as the progesterone receptor (17–19), androgen receptor (20, 21), CYP17 (22, 23), p53 (24, 25) and epoxide hydrolase (26, 27), and ovarian cancer risk. Positive associations reported by some groups have not been confirmed by others, and this likely is attributable to methodological weaknesses, including being hospital rather than population based and using controls that are poorly matched with respect to the presence of ovaries, age, and race (28). We have begun to examine candidate susceptibility polymorphisms in the context of a population-based, case control study of newly diagnosed ovarian cancer cases in central and eastern North Carolina.

Common polymorphisms in BRCA1 and 2 are high priority breast/ovarian cancer susceptibility candidates, because germ-

line mutations in these genes strikingly increase risk. In this regard, it was reported by a group in the United Kingdom that homozygosity for the H allele of the N372H polymorphism in exon 10 of BRCA2 conferred a 1.31-fold (95% CI 1.07–1.61) increased risk of breast cancer (10). It was estimated that ~2% of all breast cancers could be attributed to this polymorphism. Subsequently, a second study from Australia reported that homozygosity for the H allele was more common in cases compared with controls (9.2 *versus* 6.5%) and was associated with an elevated risk of breast cancer (OR = 1.46, 95% CI 1.05–2.07; Ref. 11).

The Australian group also examined whether N372H affects ovarian cancer risk (12). This study included 1121 ovarian cancer cases and 2643 controls from British and Australian studies. The HH genotype was associated with an increased risk of ovarian cancer in both studies, and the risk estimate for the pooled studies was 1.36 (95% CI 1.04–1.77, $P = 0.03$). There was a suggestion that this risk may be greater for ovarian cancers of the serous subtype (OR = 1.66, 95% CI 1.17–2.54, $P = 0.005$). Among 480 serous ovarian cancers, 10% were HH homozygotes compared with only 6.5% of 2643 controls.

Unlike previous studies of BRCA2 N372H that used TaqMan or allele-specific oligonucleotide assays (10–12), ours was the first study that used DNA sequencing to evaluate the genotype of all subjects. In the NCOC study, the frequency of the H allele among Caucasian controls (0.257) is essentially the same as that reported in previous studies (10–12). However, a relationship was not observed between the BRCA2 N372H polymorphism and overall risk of ovarian cancer or of the serous subtype. Although we examined >700 subjects, our study lacked sufficient power to ascertain small increases in risk, particularly in subset analyses, such as among invasive serous cases. The fact that our overall fraction of HH homozygotes was lower in cases than in controls (5 *versus* 6%, OR of 0.8; 95% CI 0.4–1.5), while not precluding the previous finding of an increased risk in cases as reported by Auranen *et al.* (3), it certainly does not lend it support.

The initial report on BRCA2 N372H suggested that there was a deficiency of HH and NN homozygotes among female controls relative to expected Hardy-Weinberg equilibrium (10). In contrast, an excess of homozygotes was observed in male controls. A deficiency of HH homozygotes was noted in newborn girls, whereas in chromosomally normal female abortuses, an excess of HH genotypes were seen. Taken together, these findings are suggestive that the H variant might affect fetal survival in a sex-dependent manner. A slight deficit of HH homozygotes was also seen in female controls in the Australian breast cancer study (50 observed *versus* 53.7 expected; Ref. 11) and ovarian cancer study (172 observed *versus* 190 expected; Ref. 29). In the current study, we found that the distribution of genotypes for BRCA2 N372H in controls conformed closely to Hardy-Weinberg equilibrium (25 HH homozygotes observed compared with 24 expected). Although this differs somewhat from the results of the previous studies discussed above, it is notable that the initial study of N372H included several control populations. A consistent excess of homozygotes was observed in several British populations, but similar to the findings in the NCOC Study, this was not the case in a Finnish population. In the ovarian cancer cases in the Finnish population, there were somewhat more NH heterozygotes than expected, rather than an excess of HH homozygotes.

Although the lifetime risk of breast cancer is similarly high in carriers of either BRCA1 or BRCA2 mutations, ovarian cancer risk is significantly higher in BRCA1 carriers. Thus, functional polymorphisms in BRCA1 might be postulated to be more likely to affect ovarian cancer risk than those in BRCA2. There are 10 polymorphisms in BRCA1 with allele frequencies >5% in Caucasians; however, only five of these (Q356R, P871L, E1038G, K1183R, and S1613G) result in amino acid changes (13). These polymorphisms, with the exception of Q356R, are in significant linkage disequilibrium and generally are inherited as part of a shared haplotype. As a result, only three halpotypes occur with a frequency of >1.3%, and the effect of all of these can be ascertained by analyzing Q356R and P871L (13, 14). Durocher *et al.* (13) examined the allele frequencies of all 10 common BRCA1 polymorphisms in a group of affected women from breast/ovarian cancer families and in control populations from Utah and Quebec. All these polymorphisms were found to be in Hardy-Weinberg equilibrium, and P871L was the only one in which a significant difference in allele frequency was seen between breast/ovarian cancer cases (L = 0.42) and controls (L = 0.28). The authors acknowledged this difference could be attributable to population admixture, because the breast/ovarian cancer cases were ascertained from many different centers. Janezic *et al.* (15) also published preliminary data consistent with an increased risk of ovarian cancer attributable to P871L from a population-based study in California in which BRCA1 sequencing was performed in women with ovarian cancer. They examined the significance of observed BRCA1 polymorphisms in 24 ovarian cancer cases and 24 sister controls. P871L was the only one in which there was a higher frequency of the L allele in cases (0.38) compared with controls (0.29), but this difference was not statistically significant.

Dunning *et al.* (14) examined the P871L polymorphism in three case control studies of breast cancer (572 total controls and 801 total breast cancers) and a hospital-based series of 237 consecutive ovarian cancers in the United Kingdom. There was no relationship between P871L genotype and risk of either breast or ovarian cancer. The frequency of the L allele was 0.32 in controls and 0.33 in ovarian cancer cases. Although no relationship with ovarian cancer risk was seen, their control group was selected to match the breast cancer cases, rather than the ovarian cancer cases. Our study represents the first population-based, case control study of BRCA1 polymorphisms in which controls were specifically matched to ovarian cancer cases with respect to important confounders such as age, race, and the presence of ovaries. We did not find an association between P871L genotype and ovarian cancer risk. As noted above with regard to BRCA2 N372H, the sample size used in our study, although relatively large, is insufficient to exclude a small increased risk attributable to BRCA1 P871L, particularly in important subsets, such as invasive serous ovarian cancers. The frequency of LL homozygotes was slightly lower in cases relative to controls, however; thus, it is unlikely that a significant OR would be achieved with a larger sample size.

African-Americans comprise >10% of our study population and have a lower incidence of ovarian cancer (ages 40–59: 17/100,000; ages ≥ 60: 24.5/100,000) relative to Caucasians

(ages 40–59: 26/100,000; ages ≥ 60: 38.4/100,000; Ref. 30). One possible explanation for the racial difference in ovarian cancer incidence may be differences in frequencies of susceptibility alleles. In this regard, we observed a striking racial difference in BRCA1 P871 allele frequencies. In Caucasians, the P allele was more common (0.64), whereas in African-Americans, the L allele predominates (0.76). This polymorphism was not associated with ovarian cancer risk in either race, however.

Previous studies of BRCA1 polymorphisms have been performed predominantly in Caucasians, however, as demonstrated above; allele frequencies may vary considerably between races. Racial variation in allele frequencies of P871L probably explains the higher L allele frequency in ovarian cancer cases relative to controls in the Durocher *et al.* study (13). Ovarian and breast cancer cases were from collected series of high-risk families, some of which likely were African-American, whereas control subjects were from geographic areas (Utah, Quebec) where few African-Americans reside. In view of the high frequency of the L allele in the African-American population, a slightly higher fraction of African-Americans among cases relative to controls would skew the distribution of allele frequencies between the groups. Racial difference between cases and controls cannot account for the association between the L allele and ovarian cancer in the Janizec study, because controls were sisters of the cases (15). However, this analysis was much too small to allow for meaningful conclusions, because it was based on only 24 cases.

The BRCA1 Q356R polymorphism segregates independently from the other common BRCA1 polymorphisms. In our large population-based, case control study, we did not find evidence to support a relationship between Q356R and ovarian cancer risk. Likewise, in the above noted study of BRCA1 polymorphisms by Durocher *et al.* (13) the rare R allele was not associated with cancer risk and had allele frequencies of ~6% in breast cancer cases, ovarian cancer cases, and controls. Dunning reported that the R allele was slightly more common among controls (7%) than breast cancer cases (6%), and RR homozygotes were only found among controls (14). Because of the rarity of the R allele, ORs were reported using a codominant model combining RR homozygotes and QR heterozygotes. The OR for breast cancer was 0.88 (95% CI 0.63–1.23), suggesting that the rare R allele might be protective against breast cancer. In examining 230 ovarian cancer cases, no relationship was seen between Q356R genotype and ovarian cancer risk. Janezic *et al.* (15) found that women in California with a family history of ovarian cancer had a higher frequency of the R allele compared with women with ovarian cancer lacking a family history. This was interpreted as consistent with an association of the R allele with ovarian cancer risk. Conversely, Smith *et al.* (16) reported that among women with ovarian cancer, the R allele of the Q356R polymorphism was more common in those who lacked a family history of cancer.

Although we did not observe associations between polymorphisms in BRCA1 or BRCA2 and ovarian cancer risk, it is possible that these polymorphisms might affect risk via gene–gene or gene–environment interactions, *e.g.,* the penetrance of deleterious BRCA mutations could be affected by polymorphisms in these same genes. Alternatively, these polymorphisms might alter risk exclusively in the setting of exposures, such as high lifetime ovulatory cycles or oral contraceptive use. We are continuing accrual in the NCOC Study and in the future hope to have sufficient power, either alone or in collaboration with other groups, to examine gene–gene and gene–environment interactions.

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Matrix Metalloproteinase-1 Gene Promoter Polymorphism and Risk of Ovarian Cancer

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OBJECTIVE: *It has been suggested that the 2G allele of a guanine insertion-deletion promoter polymorphism in the promoter of the matrix metalloproteinase-1 (*MMP1*) gene may increase susceptibility to ovarian cancer. The 2G allele also has been associated with increased* MMP1 *expression. We investigated the relationship between the* MMP1 *polymorphism and ovarian cancer risk in a large population-based, case-control study.*

METHODS: *The* MMP1 *promoter polymorphism was examined in white blood cell DNA from 311 cases and 387 age- and race-matched controls using a radiolabeled polymerase chain reaction assay. In addition, genotyping of the* MMP1 *polymorphism performed in 42 advanced-stage invasive serous ovarian cancers was compared to their mean relative* MMP1 *expression from Affymetrix microarrays.*

RESULTS: *The 2G allele frequency did not differ significantly between cases (0.49) and controls (0.48), and the distribution of genotypes was in Hardy-Weinberg equilibrium. Using 1G homozygotes as the reference group, neither 2G homozygotes (odds ratio 1.1, 95% confidence interval 0.7–1.7) nor heterozygotes plus 2G homozygotes (odds ratio 0.9, 95% confidence interval 0.7–1.3) had an increased risk of ovarian cancer. There was also no relationship between* MMP1 *genotype and histologic grade, histologic type, stage, or tumor behavior (borderline versus invasive). The mean* MMP1 *expression was twice as high in 2G homozygotes relative to 1G homozygotes, but this difference was not statistically significant.*

CONCLUSION: *The reported association between the* MMP1 *promoter polymorphism and ovarian cancer risk was not supported by our data. There was a suggestion that the 2G allele may be associated with higher* MMP1 *expression, and this finding is worthy of further investigation. (J Soc Gynecol Investig 2003;10: 381–7)* Copyright © 2003 by the Society for Gynecologic Investigation.

KEY WORDS: Matrix metalloproteinase-1, ovarian cancer, polymorphism.

It is thought that matrix metalloproteinases may play a role in the progression of some human cancers. These enzymes degrade structural components of the extracellular matrix and have been shown to enhance invasion and metastasis.[1–4] Matrix metalloproteinase-1 (*MMP1*), also known as collagenase-1, is one of a family of over two dozen matrix metalloproteinases.[2] *MMP1* is the most commonly expressed collagenase. It is produced by a variety of stromal, endothelial, and epithelial cells. Expression levels are low in most cells but can be induced by a variety of growth factors and cytokines. Conversely, cancers often exhibit high levels of constitutive *MMP1* expression.

Recently, an insertion-deletion polymorphism in the *MMP1* promoter was identified that is purported to increase *MMP1* expression.[5,6] The insertion of an extra guanine nucleotide at position −1607 bp, the 2G allele, creates a sequence (5′-GGAT-3′) that facilitates binding by members of the Ets family of transcription factors.[6] Expression of several members of the Ets family has been associated with increased *MMP1* expression and tumor aggressiveness and progression.[7–12]

The 2G allele of the *MMP1* promoter polymorphism is relatively common and has a frequency of just under 50% in the general population.[6,13,14] Association studies have been done to determine whether the *MMP1* genotype affects the risk of various types of cancers. There has been a suggestion that the 2G allele may increase lung cancer risk in smokers.[13] In addition, in a Japanese study, 89% of women with ovarian cancer were found to carry one or two 2G alleles compared with 80% of controls; this was interpreted as suggestive that the 2G allele increases ovarian cancer susceptibility.[5] In addition, ovarian cancers in women with either one or two 2G alleles had higher levels of *MMP1* expression than those with two 1G alleles. In the present study, we sought to confirm the findings

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of the Japanese study in a case-control, population-based series from the United States.

MATERIALS AND METHODS

Case-Control Study Design

Study participants were enrolled through the North Carolina Ovarian Cancer Study, an ongoing population-based, case-control study that uses rapid case ascertainment to enroll primary epithelial ovarian cancer cases. Eligible women must be 20–74 years of age with newly diagnosed ovarian cancer, reside in a 48-county region of North Carolina, and have no history of ovarian cancer before the current diagnosis. Physician permission was obtained before cases were contacted, and all information and samples were obtained with institutional review board–approved informed consent. Surgical pathology reports and tumor blocks were reviewed by an expert pathologist (R.B.) to confirm the diagnosis of primary epithelial ovarian cancer and to ascertain pathologic characteristics (stage, grade, behavior, and histology). Controls, who were frequency-matched to the cases by race (black versus nonblack) and age (5-year intervals), were from the same 48-county area of North Carolina as the cases. All eligible controls were required to have least one intact ovary. Controls were identified through either list-assisted random digit dialing or Health Care Financing Administration phone lists. Controls identified by random digit dialing were initially contacted by a telephone research company and screened for eligibility (age, county of residence, intact ovary, and ability to speak English). Those who passed the initial eligibility screening were then asked whether they would permit study personnel to contact them and provide them with additional information. After sending these potential controls a package of material describing the study, they were contacted by phone by nurse interviewers and invited into the study.

Three hundred eleven cases and 387 controls were enrolled between April 1999 and July 2001 and are included in this analysis. The study subjects were limited to white and black women. Response rates were 85% for cases and 39% for controls from the Health Care Financing Administration list. Seventy-three percent of controls identified by random digit dialing who passed the eligibility screening agreed to be contacted and sent additional study information. Among those sent additional study information the response rate was 71%. Detailed epidemiologic data were gathered by nurses during in-home interviews, and a blood sample was obtained for DNA extraction.

In-person interviews were conducted in the home of each study participant by a trained nurse interviewer using a 90-minute standardized questionnaire. We obtained information on known and suspected ovarian cancer risk factors, including family history of cancer, menstrual characteristics, pregnancy and breastfeeding history, hormone use, and lifestyle characteristics such as smoking history and exposure, alcohol consumption, talc use, sunlight exposure, physical activity, prior disease history, and occupational history. Anthropometric descriptors (height, weight, waist and hip circumference) were also measured.

Extraction of DNA

A blood sample (30 mL) was collected from each participant at the time of the interview. Within 48 hours all blood samples were centrifuged, and the buffy coat, red blood cells, and plasma were separated. Genomic DNA was obtained from leukocytes using a Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. The isolated DNA was made into a 25 ng/μL stock and stored at 4C until further analysis. There were negative controls placed within the stock plates to ensure proper plate orientation and to detect contamination. Tumor DNA was obtained from pulverized frozen specimens using the Puregene DNA Isolation Kit. Tumor samples were first evaluated microscopically to ensure the selection of tumor-enriched areas (>75% tumor cells).

Radiolabeled Polymerase Chain Reaction Genotyping Assay

Analysis of the insertion-deletion polymorphism in the *MMP1* promoter was performed as described by Kanamori et al[5] with slight modifications. Polymerase chain reaction (PCR) of white blood cell genomic DNA or cancer complementary DNA was performed in 20-μL reactions containing 1 × PCR buffer (Gibco BRL; Integrated DNA Technologies, Coralville, IA), 1.5 mM magnesium chloride, 0.5 μM sense primer 5′-GTT ATG CCA CTT AGA TGA GG-3′, 0.5 μM antisense primer 5′-TTC CTC CCC TTA TGG ATT CC-3′, 200 μM each dATP, dCTP, dGTP, and dTTP, 0.5 U Taq polymerase (Gibco BRL), and 2 μCi [α-^{32}P] dATP. The PCR cycling conditions were 95C for 3 minutes followed by 30 cycles of 95C for 45 seconds, 57C for 45 seconds, 72C for 60 seconds, followed by 72C for 10 minutes and storage at 4C until analysis.

A 3-μL aliquot of the PCR product was mixed with 2 μL of a solution containing 95% formamide, 20 mM ethylenediamenetetraacetic acid, and 0.05% bromophenol blue. The mixture was heated at 95C for 3 minutes, chilled immediately on ice, and then electrophoresed on a 6.5% polyacrylamide gel with 1× tris/boric acid/ethylenediamine tetraacetic acid (TBE) buffer at constant power of 73 W for 2.5 hours. The gel was then dried and placed for autoradiography. Bands at 148 bp and 149 bp represent the 1G and 2G alleles, respectively.

MMP1 Gene Promoter Sequencing

Confirmation of the accuracy of the radioactive genotyping was achieved by random sequencing of 10% of samples. A different pair of primers was chosen to incorporate a larger segment of the promoter region into the amplicon. This enabled more accurate evaluation of the entire PCR fragment used for radioactive genotyping. The *MMP1* promoter was sequenced after PCR in a 50-μL reaction containing 1 × PCR buffer (Gibco BRL), 1.5 mM magnesium chloride, 0.5 μM sequencing sense primer 5′-TGA CTT TTA AAA CAT

AGT CTA TGT TCA-3′, 0.5 μM sequencing antisense primer 5′-TCT TGG ATT GAT TTG AGA TAA GTC ATA GC-3′, 200 μM each of dATP, dCTP, dGTP, and dTTP, and 1.5 U Taq polymerase (Gibco BRL). The product was cleaned of primers and unincorporated bases by using a 96-well QIA Quick PCR Vacuum Purification Plate (Qiagen, Valencia, CA) followed by elution in 150 μL of 10-mM Tris-HCl, pH 8.5. One microliter of purified PCR product was mixed with 11 μL of 0.4-μM sequencing sense primer and 8 μL of Big Dye Sequencing Terminator Reaction Mix (Perkin-Elmer, Shelton, CT). PCR was performed according to supplier's recommendations and subsequently sequenced on an ABI 3100 sequencer (Perkin-Elmer).

MMP1 Expression by Microarray Analysis

Relative *MMP1* expression was obtained from data collected by Affymetrix microarray analysis of 42 invasive, stage III and IV, serous ovarian tumors. These tumors were from a bank of samples obtained with institutional review board–approved informed consent from patients treated by the Division of Gynecologic Oncology at Duke University Medical Center. RNA was extracted from approximately 30 mg of frozen ovarian cancer as described elsewhere.[15] The targets for the Affymetrix DNA microarray analysis were prepared according to the manufacturer's instructions. Targets were hybridized with the human HuGeneFL GENECHIP microarrays at 45C for 16 hours and then washed and stained using the GENECHIP Fluidics. The chips were then read with the GENECHIP scanner and the signals obtained were processed by GENECHIP Expression Analysis algorithm 3.2 (Affymetrix, Santa Clara, CA). Expression levels are represented by the average difference in hybridization intensity between the set of 20 probe pairs (perfect match − single base mismatch) for the *MMP1* gene.

Statistical Analysis

The genotype data were tested for Hardy-Weinberg equilibrium using the χ^2 goodness-of-fit test. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated with SAS software (SAS Institute Inc., Cary, NC) using unconditional logistic regression. We report crude ORs as well as adjusted ORs from a multivariate logistic regression model that included race and age. Potential confounders, including menopause status, tubal ligation, oral contraceptive use, family history of cancer, and parity, were tested individually to determine whether they changed the crude OR by 10%. Any that did were added to the multivariate model. For the overall analysis, the study had 80% power (alpha level = 0.05) to detect an OR of 1.7 or greater for carriers heterozygous for the 2G allele and an OR of 1.8 or greater for carriers homozygous for the 2G allele when compared with noncarriers. A Spearman rank correlation was used to compare zygosity with mean relative gene expression.

Table 1. Characteristics of Cases and Controls

	Cases (n = 311) n (%)	Controls (n = 387) n (%)	P
Age < 50 y	107 (34)	133 (34)	.991
Race			
White	277 (89)	337 (87)	.423
Black	34 (11)	50 (13)	
Duration of OC use (y)			
Nonusers	108 (35)	129 (34)	.061
<2	89 (29)	86 (23)	
2–5	36 (12)	52 (14)	
>5	75 (24)	115 (30)	
Missing	3	5	
Number of live births			
0	60 (19)	53 (14)	.034
1	58 (19)	61 (16)	
2	105 (34)	145 (37)	
3	52 (17)	80 (21)	
4+	36 (12)	48 (12)	
Tubal ligation	75 (24)	130 (34)	.006
Ovarian cancer in a first-degree relative	11 (4)	12 (3)	.753

RESULTS

Table 1 shows the characteristics of the 311 cases and 387 controls from the North Carolina Ovarian Cancer Study who were screened for the *MMP1* insertion-deletion promoter polymorphism. Most subjects were white (89% of cases, 87% of controls) and the remainder were black. The mean age at diagnosis or interview was 54.3 years (standard deviation [SD] 11.4 years) for cases and 55.0 years (SD 12.9 years) for controls. Although use of oral contraceptives (OCs) was similar among ovarian cancer cases compared with controls, women who had ovarian cancer used OCs for a shorter duration compared with control subjects, with age- and race-adjusted means of 5.1 years and 6.0 years of OC use, respectively (P = .06). In general, cases had a lower number of pregnancies resulting in live births (P = .03), and a lower proportion of cases compared with controls had a prior tubal ligation (24% versus 34%, respectively, P = .006). The pathologic characteristics of the ovarian cancers are shown in Table 2. Approximately three fourths of the cancers were invasive and one fourth were of

Table 2. Pathologic Characteristics of Ovarian Cancer Cases

	n (%)
Tumor behavior	
Borderline	74 (24)
Invasive	237 (76)
Stage*	
I or II	130 (42)
III or IV	180 (58)
Histologic type	
Serous	187 (60)
Endometrioid	34 (11)
Mucinous	40 (13)
Clear cell	15 (5)
Other	35 (11)

* One missing stage.

Table 3. Relationship Between MMP1 Promoter Polymorphism and Risk of Ovarian Cancer

Gene	Genotype	*n*	(%)	*n*	(%)	OR*	95% CI
		Cases		Controls			
Overall	G/G	86	(28)	101	(26)	1.0	Reference
	G/GG	147	(47)	204	(53)	0.8	0.6, 1.2
	GG/GG	78	(25)	82	(21)	1.1	0.7, 1.7
	G/GG or GG/GG	225		286		0.9	0.7, 1.3
Borderline cases		Borderline		Controls			
	G/G	23	(31)	101	(26)	1.0	Reference
	G/GG	31	(42)	204	(53)	0.6	0.3, 1.1
	GG/GG	20	(27)	82	(21)	1.0	0.5, 2.0
	G/GG or GG/GG	51		286		0.7	0.4, 1.3
Invasive cases		Invasive		Controls			
	G/G	63	(27)	101	(26)	1.0	Reference
	G/GG	116	(49)	204	(53)	0.9	0.6, 1.3
	GG/GG	58	(24)	82	(21)	1.1	0.7, 1.8
	G/GG or GG/GG	174		286		1.0	0.7, 1.4

* Adjusted for age and race.

low malignant potential. Early stage (I or II) cancers comprised 42% of cases, whereas the remainder were advanced stage (III or IV).

The distribution of *MMP1* promoter polymorphism genotypes in our control population was found to be in Hardy-Weinberg equilibrium (χ^2 = 1.249, P = .26). Allele frequencies in the controls were 52.5% for the 1G allele and 47.5% for the 2G allele, and there was no difference in allele frequencies between whites and blacks. The 2G allele frequency in cases was 49% and *MMP1* promoter polymorphism genotypes were not associated with ovarian cancer risk using either a recessive or codominant model (Table 3). Additionally, no significant difference was observed when only borderline or invasive cases were compared separately with controls. Adjusting for several additional potential confounders, including duration of OC use, family history of ovarian cancer in a first-degree relative, the number of months pregnant, and having had a tubal ligation, did not affect the magnitude of the odds ratios for the association between the *MMP-1* polymorphism and ovarian cancer. Subset analyses of serous cases (OR 1.1; 95% CI 0.5–2.4), invasive cases (OR 1.0; 95% CI 0.7–1.4), and serous invasive cases (OR 1.0; 95% CI 0.7–1.6) also did not reveal significant associations with ovarian cancer risk. When history of live birth was examined (ever versus never), the age- and race-adjusted OR for 1G2G heterozygotes was 0.4 (95% CI 0.2–0.7) for nulliparous cases versus nulliparous controls. There was no significant difference, however, when 2G homozygotes were compared with 1G homozygotes (OR 0.7; 95% CI 0.3–1.4).

We also examined whether the *MMP1* promoter polymorphism affected the pathologic phenotype of ovarian cancer. There was no difference in 2G allele frequency when comparing early (stage I or II) versus advanced (stage III or IV) cancers or borderline versus invasive cases (data not shown). There was also no relationship between *MMP1* genotype and tumor stage, histologic grade, histologic type, and history of OC use (any versus none) (data not shown).

We determined the *MMP1* promoter genotype in 42 advanced stage invasive serous ovarian cancers by both radioactive gel and direct sequencing (Figure 1). We compared *MMP1* genotypes to relative levels of *MMP1* expression from microarray data. There was a trend towards the presence of the 2G allele and increased *MMP1* expression, but this was not statistically significant (R = 0.25, P = .11). The mean expression values for 1G homozygotes, heterozygotes, and 2G homozygotes were 536 (95% CI 350–722), 792 (95% CI 520–1063), and 1027 (95% CI 537–1518), respectively (Figure 2).

DISCUSSION

The *MMP1* promoter polymorphism was originally studied in melanoma cell lines and fibroblasts. Transfection of *MMP1* promoter with the 2G sequence was found to stimulate much higher levels of transcription than the 1G allele.[6] The insertion of an extra G in the promoter creates a binding site for Ets family transcription factors, and this may explain the increased *MMP1* expression with the 2G allele. There is evidence of selection for the 2G allele during the evolution of malignant melanomas. First, the frequency of the 2G allele was shown to be increased from about 0.5 in the general population to 0.6 in melanoma cell lines.[6] In addition, the presence of the 2G allele has been associated with an invasive phenotype, and there is preferential retention of the 2G allele in melanomas that exhibit loss of heterozygosity at chromosome 11q22 where this gene resides.[16] These findings are consistent with selection for the 2G allele during the course of melanoma progression; however, case-control studies have not shown an association between the 2G allele and susceptibility to the disease.[17]

A Japanese study of the *MMP1* promoter polymorphism in ovarian cancer found that 163 cases were more likely to be either heterozygous or homozygous for the 2G allele than 150

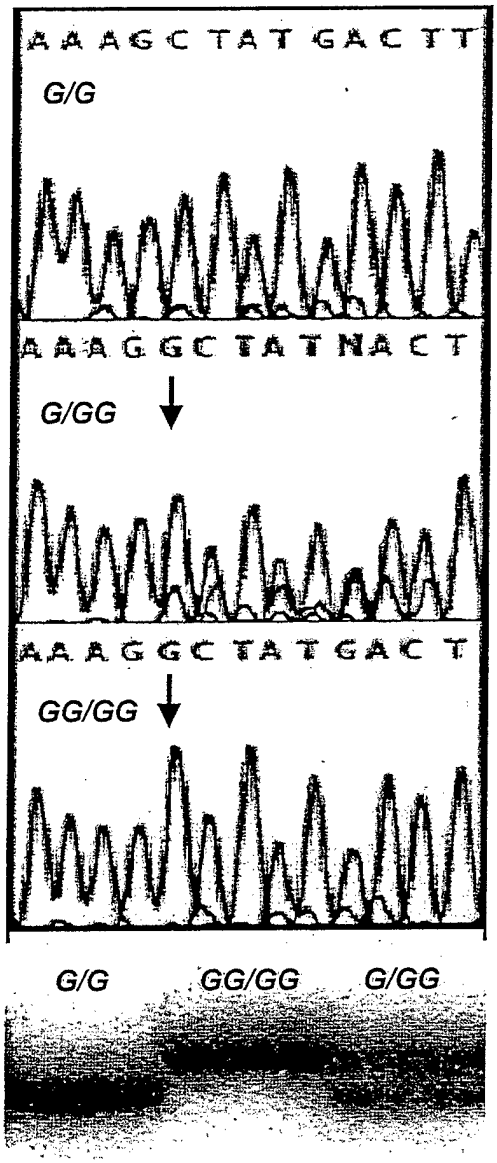

Figure 1. Genotyping of the *MMP1* polymorphism in ovarian cancers. A 1G homozygote (G/G) is shown in the top sequence and in the left lane of the autoradiograph at the bottom. A 2G homozygote (GG/GG) is shown in the bottom sequence and in the right lane on the autoradiograph. The middle sequence and lane represent a 1G/2G heterozygote (G/GG). The insertion of an extra guanine (fifth base from left) at position −1607 in the *MMP1* gene can be seen in both the heterozygote and 2G homozygote.

control subjects (89% versus 80%).[5] The frequency of 2G homozygotes was actually higher in controls (43%) than cases (37%), however. The authors hypothesized a codominant model in which inheritance of only one copy of the 2G allele is required to increase ovarian cancer risk. In support of this paradigm, they found that median *MMP1* expression of both 1G or 2G heterozygotes and 2G homozygotes was about seven-fold higher than that of 1G homozygotes. Although not stated, it appears that there was no significant difference in

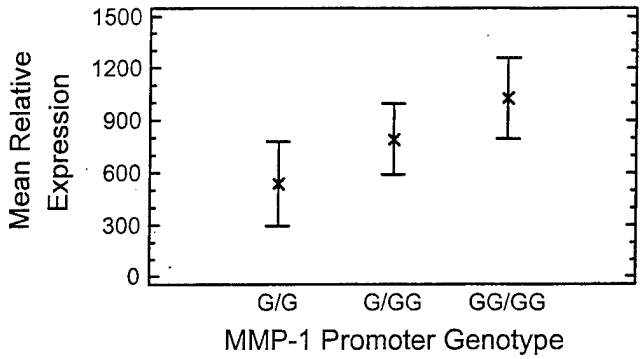

Figure 2. Relationship between *MMP1* genotype and expression. *MMP1* mean relative expression values were obtained from Affymetrix microarray analysis of 42 invasive, stage III or IV serous ovarian cancers. G/G = 1G homozygotes (n = 12), G/GG = 1G/2G heterozygotes (n = 17), and GG/GG = 2G homozygotes (n = 13). Mean sample values are indicated by an × and *whiskers* represent the 95% confidence intervals.

expression levels between 1G or 2G heterozygotes and 2G homozygotes.

The present population-based, case-control study in North Carolina did not confirm the relationship between *MMP1* promoter genotype and risk of ovarian cancer. The 2G allele frequency was 47.5% in controls and 48.5% in cases. The lack of such an association with ovarian cancer risk was apparent in both a codominant model in which 1G or 2G heterozygotes were combined with 2G homozygotes and in a recessive model in which 2G homozygotes and 1G or 2G heterozygotes were compared separately with 1G homozygotes. Subset analyses suggested a decrease of ovarian cancer for nulliparous women who were 1G or 2G heterozygotes, but this likely is a spurious finding due to multiple comparisons. In addition, the finding that heterozygotes have a decreased risk and 1G and 2G homozygotes have the same risk seems implausible.

The frequency of the 2G allele in the Japanese control population was about 15% higher (62%) than that observed in our study (47.5%).[5] This could reflect racial differences in allele frequencies; however, examination of the Japanese data showed that the distribution of genotypes in the controls was not in Hardy-Weinberg equilibrium (χ^2 = 6.85, P = .01). There was an excess of both homozygotes relative to that which would be predicted, whereas the overall 2G allele frequency was similar in cases (63%) and controls (61%). Little information was provided about the controls other than that they did not have cancer, but presumably they were all Japanese. Although it is possible that the lack of Hardy-Weinberg equilibrium could be due to unequal fitness between the alleles, the frequency of *MMP1* genotypes in our study and of others conform closely to Hardy-Weinberg equilibrium.[17] In addition, the 2G allele frequency in our study (47.5%) closely resembles that found in an Italian population (50%)[14] and a white British population (47%).[17] The Italian study compared 160 cancer cases (including 25 ovarian cancers) with 164 controls and also failed to find a significant association between 2G allele frequency and cancer risk.

The identification of polymorphisms in essentially all genes has led to a proliferation of association studies that seek to correlate specific genotypes with increased susceptibility to cancer. Several groups have examined whether polymorphisms in genes thought to be involved in ovarian carcinogenesis affect susceptibility to the disease. Positive associations have been reported for polymorphisms in the progesterone receptor,[18–20] CYP17,[12,21] p53,[22,23] epoxide hydrolase,[24,25] and others, but these findings have not been confirmed by other groups. The experience to date in ovarian cancer mirrors that seen in other cancers, with initial positive associations followed by studies that fail to confirm them.

Both positive and negative results of association studies are potentially problematic. Many published studies that have not shown an association between a polymorphism and cancer risk have not included sufficiently large populations to have the power to prove with certainty the absence of a low penetrance genetic effect. Conversely, studies that have reported positive results often have not used controls that are appropriately matched to the cases with respect to age, race, and other important characteristics. Because there is significant racial variation in allele frequencies for some polymorphisms, inconsistencies in population stratification between cases and controls can easily lead to false-positive results. In several positive association studies, such as that of the *MMP1* polymorphism in ovarian cancer,[5] the distribution of genotype frequencies in the control group was not in Hardy-Weinberg equilibrium, suggesting that differences in genotype frequencies between cases and controls are likely spurious.

MMP1 expression in cancers can derive from malignant cells or stroma, and there is evidence that stromal-tumor cell interactions are important in the regulation of *MMP1* expression. Ovarian cancers have been shown to produce extracellular matrices containing MMPs, including *MMP1*, in the absence of fibroblasts or endothelial cells.[26] Conversely, in five epithelial ovarian cancer cell lines, expression of *MMP1* was absent in four and low in one.[27] Consistent with the Japanese study,[5] we found somewhat higher *MMP1* expression in subjects with ovarian cancer and the 2G allele, which might increase its invasiveness. Because borderline tumors are noninvasive, one might expect that subjects with borderline cancers would be less likely to carry 2G *MMP1* alleles, but this was not the case. Likewise, one might expect that the 2G allele would be associated with more rapid dissemination and presentation at an advanced stage, but we did not observe such a relationship.

The relationship between the *MMP1* promoter polymorphism and risk of other cancer types has been examined. Studies in colon cancer have not provided convincing evidence of an association with risk, but they are not conclusive because of issues in study design, as addressed above.[14,28] In a relatively large study in Houston, the 2G allele was associated with increased risk of lung cancer.[13] The 2G allele frequency was 54% in controls and 63% in cases. The increased susceptibility to lung cancer was observed only in comparing cases and controls who were smokers. This study was hospital based, but controls were matched for age and race (all subjects were white). As the authors acknowledged, the fact that the distribution of genotypes in controls was not in Hardy-Weinberg equilibrium casts significant doubt on the positive association observed in that study.

In summary, the role of the matrix metalloproteinases in cancer susceptibility and progression remains a fertile area for further investigation, but the present study does not support an association between the *MMP1* promoter insertion-deletion polymorphism and risk of developing ovarian cancer or disease phenotype.

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Null Results in Brief

No Relationship between Ovarian Cancer Risk and Progesterone Receptor Gene Polymorphism in a Population-based, Case-Control Study in North Carolina[1]

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Introduction

The protective effects of pregnancy and OC[3] use on ovarian cancer risk may be attributable to the action of progestins on the ovarian epithelium (1). It has been hypothesized that a *PROGINS* is associated with increased risk of ovarian cancer. The *PROGINS* polymorphism has functional significance (2) and was associated with ovarian cancer in a pooled German/Irish population (3). A study of *BRCA1* and *BRCA2* mutation carriers found that the *PROGINS* allele was associated with a 2.4-times increased risk of ovarian cancer among the subgroup that had never used OCs (4). In contrast, no association between *PROGINS* and sporadic ovarian cancer risk has been identified in several studies with ORs ranging from 0.85 to 0.95 (5, 6). In light of these conflicting reports, we sought to investigate the hypothesis that the *PROGINS* allele is associated with increased ovarian cancer risk.

Materials and Methods

Study subjects included 309 epithelial ovarian cancer cases and 397 age- and race-matched controls enrolled through a population-based, case-control study in a 48 county region in North Carolina. Cases were 20–74 years of age at diagnosis and were identified using a rapid case ascertainment system in conjunction with the population-based North Carolina Central Cancer Registry. Controls were identified through random digit dialing and Health Care Financing Administration phone lists. The response rates for cases and controls were ~85% and 52%, respectively. Epidemiological and medical information was obtained from an in-person interview. This study has been described in more detail elsewhere (7).

Leukocyte DNA was extracted and subjected to *PROGINS* allelotyping using a PCR-based assay as described previously (5). Unadjusted and multivariable adjusted ORs and 95% CIs were calculated using unconditional logistic regression.

Results

Cases and controls were similar in age, race, education, and income. Among cases, 57% were diagnosed with stage III/IV cancer, 75% had invasive tumors, and 59% were serous.

The study had 80% power to detect an OR of $\geq$1.6 for carriers heterozygous for the *PROGINS* allele and an OR of $\geq$2.6 for carriers homozygous for the *PROGINS* allele compared with noncarriers, for risk of ovarian cancer at an $\alpha = 0.05$ level. Crude ORs for being heterozygous and homozygous for the rare allele compared with the reference group of noncarriers were 1.1 (95% CI, 0.8–1.5) and 0.8 (95% CI, 0.3–1.7), respectively (Table 1). These results remained unchanged when limiting the cases to invasive cancers only or to invasive cancers of the serous histological subtype. Adjusting for age, race, and menopause did not significantly change any of these ORs.

Within the subgroup of women who had never used OCs, we found ovarian cancer cases were more likely to have the *PROGINS* allele than controls (Table 1). When we combined homozygote and heterozygote carriers, a borderline significant increased risk was observed (adjusted OR, 1.8; 95% CI, 1.0–3.3). Among women who ever used OCs, carriers had similar risk to noncarriers (adjusted OR, 0.8; 95% CI, 0.5–1.2), although there was some suggestion of a protective effect among the subgroup homozygous for the *PROGINS* allele (adjusted OR, 0.4; 95% CI, 0.2–1.2). A statistically significant interaction between OC use and having at least one *PROGINS* allele was detected in a multivariable logistic regression model ($P = 0.04$).

Stratifying by age, parity, or race revealed no association between the *PROGINS* allele and ovarian cancer. The *PROGINS* allele was distributed similarly among those with stage I/II disease and those with stage III/IV. The *PROGINS* distribution was also similar between invasive cases with undifferentiated/poorly-differentiated cancer and those with moderately/well-differentiated tumors.

Discussion

This study supports previous negative studies and is the first population-based, case-control study to examine the relationship between the *PROGINS* allele and ovarian cancer risk. The existence of epidemiological data, as well as information on cancer stage and histology, allowed us to examine the significance of *PROGINS* within specific subgroups while controlling for potential confounding factors. The sample size of our study provides sufficient statistical power to detect an association of the level observed previously (3), but we did not observe a significant association between the *PROGINS* allele and ovarian cancer risk.

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[3] The abbreviations used are: OC, oral contraceptive; PROGINS, polymorphism in the progesterone receptor gene; OR, odds ratio; CI, confidence interval.

Table 1 ORs and 95% CIs for the association between the *PROGINS* allele in epithelial ovarian cancer cases and controls from 48 counties in North Carolina

	Genotype	Cases (n = 309)	Controls (n = 397)	Crude OR (95% CI)	OR[a] (95% CI)
		n (%)	n (%)		
Overall	T1T1	219 (71)	285 (72)	1.0 referent	1.0 referent
	T1T2	80 (26)	95 (24)	1.1 (0.8–1.5)	1.1 (0.7–1.5)
	T2T2	10 (3)	17 (4)	0.8 (0.3–1.7)	0.7 (0.3–1.6)
	T1T2/T2T2	90 (29)	112 (28)	1.0 (0.8–1.5)	1.0 (0.7–1.4)
OC users					
Yes	T1T1	146 (72)	180 (68)	1.0 referent	1.0 referent
	T1T2	51 (26)	70 (27)	0.9 (0.6–1.4)	0.9 (0.6–1.3)
	T2T2	5 (2)	14 (5)	0.4 (0.2–1.3)	0.4 (0.2–1.2)
	T1T2/T2T2	56 (28)	84 (32)	0.8 (0.6–1.2)	0.8 (0.5–1.2)
No	T1T1	73 (68)	105 (79)	1.0 referent	1.0 referent
	T1T2	29 (27)	25 (19)	1.7 (0.9–3.1)	1.7 (0.9–3.4)
	T2T2	5 (5)	3 (2)	2.4 (0.6–10.3)	2.2 (0.5–9.9)
	T1T2/T2T2	34 (32)	28 (21)	1.7 (1.0–3.1)	1.8 (1.0–3.3)

[a] ORs for the overall association are adjusted for age, race, and menopausal status. The ORs for OC use, yes or no, are adjusted for age, race, and tubal ligation.

Consistent with a previous report (4) there appeared to be some increased risk associated with the *PROGINS* allele among nonusers of OCs. However, this increased risk should be interpreted cautiously, because the *PROGINS* distribution among cases who were OC nonusers exactly matches the *PROGINS* distribution among controls who are OC users.

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